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Circulating cardiovascular microRNAs in critically ill COVID -19 patients **Short title: microRNA signatures in COVID -19**

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Abstract: Aims: Corona virus disease 2019 (COVID-19) is a still growing pandemic, causing many deaths and socio-economic damage. Elevated expression of the SARS-CoV-2 entry receptor ACE2 on cardiac cells of patients with heart diseases may be related to cardiovascular burden. We have thus analysed cardiovascular and inflammatory microRNAs (miRs), sensitive markers of cardiovascular damage, in critically ill, ventilated patients with COVID-19 or Influenza associated acute respiratory distress syndrome (Influenza-ARDS) admitted to intensive care unit (ICU) and healthy controls. **Methods and results:** Circulating miRs (miR-21, miR-126, miR-155, miR-208a and miR-499) were analyzed in a discovery cohort consisting of patients with mechanically-ventilated COVID-19 (n = 18) and healthy controls (n = 15). A validation study was performed in an independent cohort of mechanically-ventilated COVID-19 patients (n = 20), Influenza-ARDS patients (n = 13) and healthy controls (n = 32). In both cohorts RNA was isolated from serum and cardiovascular disease/inflammatory-relevant miR concentrations were measured by miR-specific TaqMan PCR analyses. In both the discovery and the validation cohort, serum concentration of miR-21, miR-155, miR-208a and miR-499 were significantly increased in COVID-19 patients compared to healthy controls. Calculating the area under the curve (AUC) using ROC-analysis miR-155, miR-208a and miR-499 showed a clear distinction between COVID-19 and Influenza-ARDS patients. **Conclusion:** In this exploratory study, inflammation and cardiac myocyte-specific miRs were upregulated in critically ill COVID-19 patients. Importantly, miR profiles were able to differentiate between severely ill, mechanically-ventilated Influenza-ARDS and COVID-19 patients, indicating a rather specific response and cardiac involvement of COVID-19. **Keywords:** ARDS; COVID-19; SARS-CoV-2; biomarker; influenza; microRNA.

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Short title: microRNA signatures in COVID-19

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Keywords: COVID-19, SARS-CoV-2, ARDS, influenza, microRNA, biomarker

Abstract

Aims

Corona virus disease 2019 (COVID-19) is a still growing pandemic, causing many deaths and socio-economic damage. Elevated expression of the SARS-CoV-2 entry receptor ACE2 on cardiac cells of patients with heart diseases may be related to cardiovascular burden. We have thus analysed

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cardiovascular and inflammatory microRNAs (miRs), sensitive markers of cardiovascular damage, in critically ill, ventilated patients with COVID-19 or Influenza associated acute respiratory distress syndrome (Influenza-ARDS) admitted to intensive care unit (ICU) and healthy controls.

Methods and Results

Circulating miRs (miR-21, miR-126, miR-155, miR-208a and miR-499) were analyzed in a discovery cohort consisting of patients with mechanically-ventilated COVID-19 (n=18) and healthy controls (n=15). A validation study was performed in an independent cohort of mechanically-ventilated COVID-19 patients (n=20), Influenza-ARDS patients (n=13) and healthy controls (n=32). In both cohorts RNA was isolated from serum and cardiovascular disease/inflammatory-relevant miR concentrations were measured by miR-specific TaqMan PCR analyses.

In both the discovery and the validation cohort, serum concentration of miR-21, miR-155, miR-208a and miR-499 were significantly increased in COVID-19 patients compared to healthy controls. Calculating the area under the curve (AUC) using ROC-analysis miR-155, miR-208a and miR-499 showed a clear distinction between COVID-19 and Influenza-ARDS patients.

Conclusion

In this exploratory study, inflammation and cardiac myocyte-specific miRs were upregulated in critically ill COVID-19 patients. Importantly, miR profiles were able to differentiate between severely ill, mechanically-ventilated Influenza-ARDS and COVID-19 patients, indicating a rather specific response and cardiac involvement of COVID-19.

Introduction:

Corona virus disease 2019 (COVID-19) is still a rapidly growing pandemic disease, which has claimed already more than 1.5 million lives worldwide. Despite massive efforts by the scientific community and enormous accumulation of new insights, the complexity of COVID-19 caused by the novel corona virus SARS-CoV-2 is still far from being completely understood. Infection with this β -genus SARS-CoV-2 can lead to the potentially lethal COVID-19, primarily characterized by pneumonia which can progress into acute respiratory distress syndrome (ARDS). A relatively high incidence of cardiac complications along with respiratory manifestations in COVID-19 patients has raised concerns, and might be significantly contributing to overall mortality¹⁻³. Moreover, multiple organ dysfunctions in infected patients might be triggered and aggravated by a maladaptive immune response that causes systemic inflammatory response associated with endothelial dysfunction⁴⁻⁷.

As per the initial understanding, patients with pre-existing cardiovascular diseases are at higher risk for severe courses of COVID-19 presumably related to higher susceptibility to endothelial injury due to their pre-existing vascular burden or higher expression of its entry receptor ACE2 in cardiovascular cells including myocytes, fibrocytes and endothelial cells^{8,9}. The spectrum of cardiovascular manifestations ranges from elevated markers of cardiovascular damage such as troponins to severe arrhythmias and even myocardial infarctions^{3,10}. Therefore, analysing markers pertaining to cardiovascular properties in COVID-19 patients might be helpful in predicting unfavourable outcome and complications.

MicroRNAs (miRs) are short noncoding ribonucleic acids which play essential regulatory roles in various cellular functions and disease processes. Moreover, their ease of detection in our circulatory system has established them as potential sensitive disease biomarkers especially in the cardiovascular context¹¹⁻¹⁵. Therefore, we sought to identify changes in selected miRs; cardiovascular disease and inflammation-associated (miR-155), myocyte-associated miRs (myomiRs miR-499 and miR-208a), fibromiRs (miR-21) and endothelial miRs (miR-126) in the blood from mechanically-ventilated ICU patients with COVID-19 associated pneumonia in comparison to Influenza-ARDS patients and healthy controls.

Methods

Clinical data:

Clinical data (demographics, cardiovascular risk factors, cardiac premedication, laboratory, organ function scores at time of sampling, clinical outcomes) and serum were obtained from ICU patients with severe COVID-19 pneumonia requiring invasive ventilation (one patient received high flow nasal oxygen therapy at time of sampling but was intubated shortly after) and severe, mechanically-ventilated influenza ARDS patients (Influenza-ARDS group). The investigation conforms to the principles outlined in the 1964 Declaration of Helsinki and later amendments¹⁶. All patients or their health representatives provided written consent. Healthy volunteers were chosen as controls. The study was approved by the local institutional review board (#8164_BO_K_2018 and #8980_BO_K_2020).

RNA isolation:

RNA was isolated from 100 μ L of serum using the miRNeasy Serum/Plasma Kit (Qiagen, as described by the manufacturer. Synthetic cel-miR-39-3p (1.6×10^8 copies/ μ L) (Qiagen) was spiked-in before RNA isolation for normalization in subsequent quantitative real-time PCR (qRT-PCR). RNA was eluted from the columns with 30 μ L of water^{12,17}. The RNA was treated with heparinase to remove traces of heparin known to interfere with PCR reactions.

PCR-based analysis of miRs:

The isolated RNA was reverse transcribed to complementary DNA (cDNA) utilising TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) following manufactures guidelines. RT-qPCR was performed with specific TaqMan miR assays (Applied Biosystems®) for Cel-miR-39, hsa-miR-21-5p, hsa-miR-126-3p, hsa-miR-155-5p, hsa-miR-208a-3p and hsa-miR-499-5p on a Viia7 system (Thermo Fisher Scientific). miRs with $C_q \geq 36$ were deemed to be undetected and set to $C_q = 36$. Relative miR concentrations were calculated using the 2^{-dC_q} method ($dC_q = C_q[\text{miR}] - C_q[\text{Cel-miR-39}]$). Relative miR levels were log2 transformed¹².

Statistical analysis:

Statistical analysis of miR concentrations between the two groups was performed via Mann-Whitney test in the discovery cohort. In the validation cohort Dunn's multiple comparisons test was used to compare healthy controls, Influenza-ARDS and COVID-19 patients. For group discrimination by levels of selected miRs receiver operating characteristic (ROC) curves were used and the area under the curve (AUC) was calculated. Clinical characteristics depending on miR levels were compared by Chi² or Wilcoxon-U-Test, as appropriate after subgrouping by median concentration of individual miRs. All analyses and graph production were performed using STATA V16.1 (USA) and GraphPad Prism 9 (USA).

Results

In this exploratory study we initially compared serum miR concentrations in critically ill COVID-19 patients with that of healthy individuals as a discovery cohort. The control group consisted of 15 subjects (median age 31 years, 93% male) while the COVID-19 group comprised 18 subjects (median age 59 years, 94% male). Demographic data of discovery cohort are described in (**Table 1, online data supplement**). In the discovery cohort miR-21, miR-155, miR-208a and miR-499 were significantly upregulated in COVID-19 patients compared to healthy controls whereas miR-126 levels were significantly decreased (**Figure 1**).

For validation, we recruited an independent cohort of mechanically-ventilated COVID-19 patients (n=20, median age 59.5 years, 70% male), as well as invasively-ventilated Influenza-ARDS patients (n=13, median age 56 years, 85% male) with similar respiratory compromise (**Table 1**) and reasonably age-matched healthy controls (n=32; median age 50 years; 62.5% male). When comparing the cardiovascular risk factors between the COVID-19 and Influenza-ARDS groups, with the exception of smoking history no significant differences in the number of diabetic, arterial hypertension, dyslipidemia and obesity were observed (**Table 1**). No significant differences in cardiac premedication (use of ACE inhibitors, AT1 inhibitors, beta blockers, diuretics, calcium antagonists and aldosterone antagonists) were found (**Table 1**). Procalcitonin, LDH, Troponin-T, Creatine kinase and SOFA score were significantly higher in the Influenza-ARDS group compared to COVID-19 patients. The median noradrenaline dose was higher in the Influenza-ARDS group (**Table 1**).

In line with the validation cohort, miR-21 was increased in the COVID-19 group compared to healthy controls and to Influenza-ARDS patients (**Figure 1&2 A**). Changes in miR-126 concentration could not be confirmed in the validation cohort (**Figure 1&2 B**). Upregulation of miR-155 and miR-499 in COVID-19 patients was confirmed in the second cohort both compared to healthy controls and Influenza-ARDS patients (**Figure 1&2 C&E**), whereas miR-208a was upregulated in both severe COVID-19 and Influenza-ARDS group compared to healthy controls (**Figure 1&2 D**).

ROC analyses of inflammation and cardiac associated miRs miR-155, miR-208a and miR-499 showed a strong discrimination between COVID-19 and Influenza-ARDS by these markers of myocardial damage (AUC: 1.000 (miR155); 0.796 (miR-208a); 0.865 (miR-499)) (**Figure 3**).

Stratification into low and high miR concentrations divided by the median concentration revealed an association of lower miR-21 levels with more ventilation and vasopressor days as well as a higher rate of ECMO and renal-replacement therapy (**Table 2**). Higher miR-208a and miR-499 levels were associated with elevated procalcitonin and lactate (**Table 2**).

Discussion

In this exploratory study, we aimed to investigate altered levels of circulating cardiovascular and inflammatory miRs in severe COVID-19 patients requiring invasive ventilation to identify biomarkers for myocardial damage and disease severity. The rationale for the design of this study was the well-described association of miR-155 with inflammation, miR-208a and miR-499 with myocardial/cardiomyocyte damage, and miR-21 and miR-126 with cardiac fibroblast and endothelial cell dysfunction, respectively¹⁸. To our knowledge, this is the first study reporting the interlink of miR levels in blood of COVID-19 patients and clinical parameters. Our study identified significantly altered levels of cardiac-associated miRs in COVID-19 patients indicating a strong association of COVID-19 with cardiovascular ailments and respective biomarkers.

In order to strengthen the quality and significance of the exploratory study, we performed our analysis across two waves of the COVID-19 pandemic to include a broader pool of patient samples. We initially analyzed a discovery cohort during the first wave of COVID-19 and then, after recruiting new patients with the emergence of a second wave of COVID-19, a validation study was performed.

Except for miR-126, all miRs were consistently upregulated in the COVID-19 group compared to the healthy controls in both the discovery and the validation cohort. In line with the clinical picture of the disease, inflammatory miRs, such as miR-155¹⁹ and heart-muscle specific miRs, also called myomiRs, such as miR-208a²⁰ and miR-499^{20,21} were significantly upregulated in COVID-19 patients. Interestingly, the altered levels of miR-155 and miR-499 could further distinguish between COVID-19 and Influenza-ARDS groups, even though both the diseases are phenotypically very similar. With the levels of inflammation associated miR-155 being highest at the site, the markedly elevated levels in COVID-19 might be reflective of the since widely-appreciated endothelialitis, which is COVID-19-specific²². In line with this, miR-126 which is known to be protective from endothelial damage and deprived in chronic cardiovascular disease²³ was decreased in our discovery cohort, albeit this finding was not reproducible in the validation cohort. Within the COVID-19 group, the patients with higher miR-208a and miR-499 also had a higher Procalcitonin and lactate levels. However, this clinical correlation should not be overestimated as Procalcitonin and lactate were relatively low on average. A correlation between deregulated miRNA levels and ECMO support has also been observed previously, however, whether ECMO support caused the miR deregulation or miR deregulation called for ECMO support is not very clear²⁴. In this regard, it has been demonstrated that the use of ECMO is not associated with significant changes in circulating miRs within 24 hours before and after ECMO-implantation²⁵.

Interestingly, fibrosis-associated miR-21¹¹ was found to be increased in acute COVID-19 compared to healthy controls and Influenza-ARDS patients. The upregulation of miR-21, miR-155, miR-208a and miR-499 in COVID-19 survivors might be a predictor of chronic myocardial damage and inflammation. Of note, despite troponin levels being higher in the Influenza-ARDS group, myocardial specific miR-208a and miR-499 were more upregulated in COVID-19. In this context, a recent cardiovascular magnetic resonance (CMR) imaging study in patients recently recovered from SARS-CoV2 infection provided alarming results. In 78 out of 100 patients CMR abnormalities were found and 60 patients presented with ongoing myocardial inflammation while high sensitive troponin levels were only elevated in 5% of the cohort²⁶, indicating troponin is not a useful marker of myocardial remodeling processes in COVID-19. Importantly, these findings were independent of

preexisting conditions and severity and overall course of COVID-19²⁶. This highlights the need for easily accessible biomarkers, such as circulating miRs, which may provide predictive information on potential long-term cardiovascular consequences. Overall, we believe that the significance of this study is strengthened by the fact, that patients in both the COVID-19 group and the Influenza-ARDS group had a similar cardiac pre-existing disease spectrum prior to hospitalization, but the miR concentration is significantly different in severe ARDS. This confirms that unlike influenza, which mainly triggers a pulmonary response, COVID-19 infection triggers multi-organ involvement with inflammation, endothelial cell damage and cardiac involvement.

Although this study encompasses a rather small patient population, our exploratory approach divided in two independent cohorts provides important insights into the pathophysiological aspects of miRs in COVID-19. These should serve as a foundation for larger studies and also serve to analyze the long-term course of COVID-19 patients.

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Author Contribution Statement

A.G., A.Gi. and K.S. performed the experiments; A.A.D., B.S. and K.So. prepared demographic data. A.A.D., A.G., B.S., C.B. and K.C. analysed the data; A.A.D., A.G., B.S., C.B.,

S.D. and T.T. interpreted the data for the work; A.A.D., B.S. and C.B. prepared figures; A.A.D., A.G., B.S., C.B., S.D. and T.T. drafted the manuscript; A.A.D., A.G., B.S., C.B., I.P., J.B., M.M.H., S.D., T.T. and T.W. contributed to the study design and revised manuscript; A.A.D., A.G., B.S., C.B., S.D., and T.T. approved final version of manuscript; C.B., S.D. and T.T. designed research.

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Conflict of Interest

T.T. and J.B. filed and licensed patents in the field of non-coding RNAs (outside the here submitted work). T.T. is founder and holds shares of Cardior Pharmaceuticals GmbH (outside the here submitted work). J.B. reports personal fees from Abbott, grants and personal fees from Abiomed, personal fees from Astra Zeneca, personal fees from Bayer, personal fees from BMS, personal fees from Boehringer Ingelheim, grants and personal fees from CvRX, personal fees from Daiichi Sankyo, personal fees from Medtronic, personal fees from MSD, personal fees from Novartis, personal fees from Pfizer, personal fees from Servier, grants and personal fees from Vifor, grants from Zoll, outside the submitted work. C.B. filed and licensed patents in the fields of telomerase and non-coding RNA therapy (outside the here submitted work). M.M.H. reports personal fees from Actelion, personal fees from Bayer, personal fees from Acceleron, personal fees from GSK, personal fees from Janssen, personal fees from MSD, personal fees from Pfizer, outside the submitted work. I.P. reports non-financial support from TEVA, non-financial support from Chiesi, outside the submitted work. All other authors have nothing to disclose.

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Figure Legend

Figure 1. Levels of the indicated miRs in healthy controls and COVID-19 patients. Data are presented as dot plots and mean \pm standard deviation (SD). Mann-Whitney test was used for statistical comparison.

Figure 2. Levels of the indicated miRs in healthy controls, Influenza-ARDS and COVID-19 patients. Data are presented as dot plots and mean \pm standard deviation (SD). Dunn's multiple comparisons test was used for statistical comparison.

Figure 3. ROC curve analysis of multiple microRNAs (miR-155, miR-208a, miR-499) was used to distinguish between the COVID-19 and the Influenza-ARDS group.

Table 1. Demographics Validation Cohort

Characteristics	COVID-19 (n=20)		Influenza-ARDS (n=13)		p-value
Age (y), median (IQR)	59.5	(46-68)	56	(49-58)	0.554
Male (%)		14 (70)		11 (85)	0.338
Body mass index (kg/m ²), median (IQR)	29.4	(25.7-33.4)	25	(24.5-29.2)	0.107
Cardiovascular risk factors					
Diabetes (%)		6 (30)		0 (0)	0.060
Arterial hypertension (%)		11 (55)		5 (38)	0.481
Dyslipidemia (%)		3 (15)		4 (31)	0.393
Obesity (%)		5 (25)		4 (31)	>.999
Ever smoker (%)		0 (0)		6 (46)	0.002
Cardiac premedication					
ACE inhibitor (%)		3 (15)		1 (8)	0.638
AT1 inhibitor (%)		2 (10)		3 (23)	0.360
Beta blocker (%)		5 (25)		3 (23)	>.999
Diuretic (%)		4 (20)		1 (8)	0.625
Calcium antagonist (%)		2 (10)		2 (15)	>.999
Aldosterone antagonist (%)		0 (0)		1 (8)	0.394
Basal echocardiographic data					
No. of existing data (%)		7 (35)		8 (62)	
Preserved LVEF (%)		7 (100)		8 (100)	>.999
LV hypertrophy (%)		3 (43)		3 (38)	>.999
Laboratory at time of sampling					
CRP (mg/L), median (IQR)	123	(92-197)	197	(141-296)	0.069
Procalcitonin (ng/L), median (IQR)	0.45	(0.2-1.0)	3.55	(1.55-9.55)	<.001
IL-6 (ng/L), median (IQR)	98	(32-304)	-		
Ferritin (µg/L), median (IQR)	1513	(1065-2267)	1613	(620-5017)	0.910
Leukocytes (gpt/L), median (IQR)	8.8	(7.1-16.4)	8.6	(6.5-12.6)	0.407
Lactate (mmol/L), median (IQR)	1.7	(1.3-2.0)	1.2	(1.0-1.8)	0.154
D-Dimers (mg/L), median (IQR)	1.24	(0.8-3.7)	1.8	(1.1-4.4)	0.325

LDH (U/L), median (IQR)	433	(358-572)	550	(515-908)	0.004
NT-pro-BNP (ng/L)	482	(278-803)	1211	(380-2611)	0.068
Troponin T (ng/L), median (IQR)	12	(6-25)	42	(14-110)	0.011
Creatine kinase (U/L), median (IQR)	301	(97-589)	918	(721-1846)	0.003
p _a O ₂ / FiO ₂ (mmHg), median (IQR)	97	(64-153)	83	(60-100)	0.137
SOFA score at day of sampling (IQR)	8	(6-9)	11	(10-12)	0.001
Treatment modalities at time of sampling					
Invasive ventilation (%)		18 (90)		13 (100)	0.239
PEEP (mbar), median (IQR)	14	(10-16)	15	(14-16)	0.483
P _{max} (mbar), median (IQR)	28	(25-30)	26	(25-28)	0.640
ECMO (%)		3 (15)		5 (38)	0.124
Vasopressor use (%)		14 (70)		12 (92)	0.423
Noradrenaline dose (µg/kg/min), median (IQR)	0.175	(0.057-0.267)	0.306	(0.212-0.563)	0.030
Treatment modalities and outcomes during ICU stay					
Renal replacement therapy (%)		6 (30)		8 (62)	0.073
ECMO (%)		8 (40)		7 (54)	0.435
ECMO days, median (IQR)	9	(7-15)	12	(3-20)	0.799
ICU days, median (IQR)	18	(12-26)	18	(15-21)	0.920
Vasopressor days, median (IQR)	8	(3-12)	8	(4-13)	0.792
Ventilation days, median (IQR)	13	(8-20)	15	(9-17)	0.736
28-day ICU mortality (%)		2 (10)		5 (38)	0.051

y – years; IQR – interquartile range; ACE – Angiotensin-converting enzyme; AT1 – angiotensin II receptor type 1; No. – number; IL-6 – interleukin-6; CRP – C-reactive protein; p_aO₂ – arterial partial pressure of oxygen; FiO₂ – Fraction of inspired oxygen; SOFA score – sequential organ failure assessment score; PEEP – positive end expiratory pressure; P_{max} – pressure maximum; ECMO – extracorporeal membrane oxygenation; ICU – intensive care unit.

Healthy control subjects were part of the validation cohort (n=32, median age 50 y, 62.5% male).

Table 2. Differences in clinical and laboratory data at time of sampling between COVID-19 patients of validation cohort with high/low* levels of the investigated miRs.

Variable	miR-21 ^{low}	miR-126 ^{low}	miR-155 ^{high}	miR-208a ^{high}	miR-499 ^{high}
	p-value (p<0.1; p<0.05; p<0.01); ↑ / ↓=increased / decreased in reference group				
ICU outcomes					
Ventilation days	↑ 0.040	0.422	0.752	0.269	0.563
Vasopressor days	↑ 0.015	0.183	0.727	0.644	0.644
Vasopressor dose	0.259	0.209	0.620	0.755	0.606
Laboratory					
IL-6	0.128	0.197	0.305	0.239	0.149
CRP	↑ 0.096	↑ 0.082	0.353	0.393	0.481
PCT	0.790	0.889	0.961	↑ 0.061	↑ 0.026
Ferritin	0.199	0.940	0.740	0.880	0.199
Thrombocytes	0.542	0.565	0.469	0.494	0.542
Bilirubin	0.826	0.645	0.983	0.617	0.535
D-Dimer	0.360	0.573	0.762	0.122	0.136
Lactate	0.616	0.671	0.270	↑ 0.007	↑ 0.030
Troponin T	0.855	0.233	↓ 0.060	0.829	0.537
Creatine kinase	0.218	0.123	0.739	0.105	0.436
NT-Pro-BNP	0.971	0.684	0.280	0.684	0.579
LDH	↓ 0.049	↓ 0.082	1.000	0.631	1.000
Treatments at any time					
Dialysis	↑ 0.051	0.329	0.329	0.329	1.000
ECMO	↑ 0.006	↑ 0.068	0.361	1.000	1.000